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Hepatocyte steatosis is a cytopathic effect of hepatitis C virus genotype 3

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Background/Aims: **Patients infected with the hepatitis C virus (HCV) often have liver steatosis, suggesting the possibility of a viral cytopathic effect. The aim of this study was to correlate the occurrence and severity of liver steatosis with HCV RNA type, level and sequence of the core-encoding region.**

Methods: **We scored the liver steatosis in 101 HCV-infected individuals carefully selected to exclude other risk factors of a fatty liver. Results were compared with HCV RNA genotype and level in serum and liver. In selected patients, we assessed the effect of antiviral therapy on steatosis and the relationship between nucleocapsid sequence heterogeneity and fat infiltration.**

Results: **Steatosis was found in 41 (40.6%) patients, irrespective of sex, age or route of infection. HCV genotype 3 was associated with higher steatosis scores than other genotypes. A significant correlation between steatosis score and titer of intrahepatic HCV RNA was found in patients infected with genotype 3, but not in those infected with genotype 1. In selected patients, response to alpha-interferon was associated with the disappearance of steatosis. Analysis of the nucleocapsid of 14 HCV isolates failed to identify a sequence specifically associated with the development of steatosis.**

Conclusions: **We provide virological and clinical evidence that the steatosis of the liver is the morphological expression of a viral cytopathic effect in patients infected with HCV genotype 3. At variance with published evidence from experimental models, the HCV nucleocapsid protein does not seem to fully explain the lipid accumulation in these patients.**

Key words: **Liver steatosis; Sequence heterogeneity; Viral cytopathic effect; Viral pathogenesis.**

The hepatitis C virus (HCV) is a common human pathogen, its persistent infection being mostly associated with chronic hepatitis and leading to cirrhosis in about 20% of patients over a 20-year follow-up (1). A peculiar histopathological feature associated with chronic hepatitis C is steatosis of the liver (2–6). The proportion of patients infected with HCV who have a fatty liver may be as high as 50%. The causes underlying the fat accumulation in chronic hepatitis C patients may encompass obesity, drugs, alcohol, diabetes and concomitant infections. However, even when all of these causes are carefully excluded, a significant proportion of patients persistently infected with HCV may still have a fatty liver (7,8). This feature is so frequent among chronic hepatitis C patients as compared to patients with other types of chronic hepatitis that it is considered of diagnostic significance (7).

These observations suggest that the lipid accumulation in hepatocytes may be due to a direct cytopathic effect of HCV (6,8). However, there are no studies that have analyzed the correlation between the level of HCV replication and the degree of fat accumulation. A single *in situ* hybridization-based study showed the occasional presence of HCV RNA in steatotic hepatocytes, but this association was not studied in detail (9). Another study suggested a link between the liver steatosis and HCV genotype 3 (6), but risk factors known to cause fatty liver, other than HCV, were not carefully excluded. Experiments conducted *in vitro* (10) and in transgenic mice (11) have suggested that the nucleocapsid protein of HCV may be involved in the patho-

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genesis of the lipid accumulation, but these data have not been confirmed as yet in the human infection.

We recently reported an assay to titer both strands of HCV RNA (i.e. the genomic RNA and the putative replication intermediate or minus-strand RNA) in liver tissue (12,13). Thus, we analyzed the relationship between the HCV level of replication (in both serum and liver) and the severity of steatosis in a population of hepatitis C patients without other risk factors for a fatty liver. To further investigate whether HCV may directly be involved in the lipid accumulation, we studied its association with the viral genotype, the response to antiviral treatment, and with the liver-derived, HCV nucleocapsid aminoacid sequence of some selected, representative viral isolates.

Materials and Methods

Patient populations

A total of 101 patients were studied. A first group consisted of 70 immunocompetent patients with persistent HCV infection, as documented by the presence of HCV RNA in serum detected by a 2nd generation qualitative RT-PCR assay (Amplicor™, Roche, Switzerland; sensitivity limit 100 copies/ml) for at least 6 months, and histologically-proven chronic hepatitis. There were 38 males and 32 females. The median age was 44.1 years (range 19–67). All patients lacked factors likely to be associated with the development of a fatty liver, i.e. chronic alcohol consumption, body mass index (weight [kg] divided by height^2 [m^2]) greater than 26, elevated serum level of cholesterol or triglycerides, diabetes or the administration of potentially hepatotoxic drugs (8,14,15). Twenty-one patients received a course of treatment with α-interferon (α-IFN). Among the nine with an initial virological response (i.e. undetectable serum HCV RNA after 3 months of therapy), three maintained the response for at least 1 year after the end of treatment and were considered as sustained responders.

A second group consisted of 31 patients with end-stage HCV-related cirrhosis who underwent an orthotopic liver transplant (OLT). There were 23 males and eight females. Their mean age at OLT was 51 years (range 36–64 years). Like the above immunocompetent patients, transplanted patients had no risk factors for liver fatty infiltration at the time they received the liver graft or thereafter. After OLT, they were followed for a median of 31 months (range 2–116 months). All patients received a standard triple immunosuppression, consisting of prednisone 100 mg on the first day post-OLT, tapered to 20 mg daily on day 10 and maintained for 6–12 months, azathioprine 1–2.5 mg/kg daily for 6–12 months, and cyclosporine A 5–10 mg/kg per os daily. A total of 20 patients who underwent an OLT for a HCV- and hepatitis B virus-negative liver disease served as controls. The latter patients were followed for a median of 66 months (range 23–121 months) after OLT and received an immunosuppression regimen comparable to that of HCV-positive patients. Indications for OLT were primary biliary cirrhosis (10 cases), non-viral fulminant liver failure (3 cases), alcoholic cirrhosis (2 cases), type I familial amyloidosis (Portuguese variant) (2 cases), and diffuse nodular hyperplasia, Byler's disease, Wilson's disease (1 case each).

All patients gave written informed consent to the study. All clinical trials considered in the present study were approved by our Institutional Review Board.

Liver histology

A liver biopsy was obtained from all patients. OLT patients underwent a liver biopsy at every clinically significant ALT elevation, or once a year if ALT was normal.

Liver specimens were formalin-fixed and paraffin-embedded for histological evaluation. Histological diagnoses were established according to internationally accepted criteria (16). H&E-stained sections were evaluated according to a scoring system which includes the semi-quantitative assessment of liver disease grading and staging (16). Macrovesicular steatosis was graded as absent or minimal (less than 1% of hepatocytes) (score 0), mild (<30% hepatocytes involved) (score 1), moderate (between 30 and 60% of hepatocytes involved) (score 2) or severe (>60% of hepatocytes involved) (score 3) (17). The presence of microvesicular steatosis and lipogranulomata was also recorded. Perl's stained slides were evaluated for the presence and extent of iron deposits by a semi-quantitative score, the histochemical hepatic iron index (HHII) (18). A correction factor was applied to the HHII whenever required by a heterogenous iron lobular distribution (19).

Semi-quantitative RT-PCR for genomic- and minus-strand HCV RNA

A specimen adjacent to the one being processed for routine histological studies was obtained in all 70 immunocompetent patients and from 69 liver biopsies taken from the 31 HCV-positive OLT patients, snap-frozen in liquid nitrogen and stored at −80°C. Total liver RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform procedure (20). A strand-specific RT-PCR assay was used to assess the presence and relative titers of either HCV RNA strand (12,13). Semi-quantitation was achieved by performing a nested RT-PCR to the endpoint on 2- to 4-fold dilutions (in 10 μg/ml of *Escherichia coli* tRNA [Sigma]) of an initial amount of 100 ng of total liver RNA. Titers were expressed as the last dilution giving a visible band of the appropriate size on a 1.6% agarose gel stained by ethidium bromide. Intrahepatic genomic- and minus-strand HCV RNA titers were normalized to an arbitrary β-actin mRNA titer of 1024, as measured on the same specimen (21). The strand-specificity, sensitivity and reproducibility of the RT-PCR detection of liver HCV RNA have been reported before (12). In particular, this assay was shown to detect as few as 15 copies of each HCV RNA strand per assay, in a genotype-independent way (13).

Serum assays

Serum samples were collected at the time of the liver biopsy and stored at −80°C until use. Assays included detection of HCV RNA by qualitative RT-PCR (Amplicor™, Roche, Switzerland) or by a quantitative signal amplification-based, branched DNA assay (bDNA) (Quantiplex™ version 2, Chiron Corp., Emeryville, CA, USA). HCV RNA genotyping was performed by restriction fragment length polymorphism of sequences amplified in the 5′ non-coding region (22). The hepatitis G virus (HGV) RNA was extracted from 25 μl of serum and assayed by a modified RT-PCR procedure (23,24).

Sequence analysis of HCV RNA

Total liver RNA from selected cases was reverse transcribed using random hexamers and AMV reverse transcriptase (Promega Biotech, Madison, WI, USA). The cDNA was then amplified by two 35-cycle rounds of PCR using 1.25 U *Pfu* polymerase (Promega) and primers specific for genotype 1b and 3a, encompassing the entire HCV nucleocapsid-encoding region (base positions −24 through 597 [genotype 1b] or 602 [genotype 3a]). The primers used in both PCR amplifications have the following sequence: primer 5′UTRS (sense primer for both genotype 1b and 3a) 5′-GGG AGG TCT CGT AGA CCG TG-3′; primer 1bE1AS (antisense primer specific for genotype 1b); 5′-CCC GGA CGC GTT GCG CAC TT-3′; and primer 3aE1AS (antisense primer specific for genotype 3a) 5′-TAG AGG CCG GAC GTG TTC CG-3′. Annealing temperatures were 60°C and 52°C for the first and second amplification, respectively. PCR products were purified on 2% low-melting agarose electrophoresis gel with the Wizard DNA PCR Preps purification system (Promega) and directly sequenced using standard protocols for the ABI 377 automated sequencer. Primers used for the sequencing reactions (both directions) were the same as used for the two PCR amplifications. Sequences were analyzed by multiple sequence alignment with hierarchical clustering (25).

TABLE 1

Characteristics of 70 chronic hepatitis C patients with or without steatosis of the liver

	With steatosis (n=28)	Without steatosis (n=42)	p
Sex (male/female)	15/13	23/19	NS
Age	44.1±10.5	42.5±10.7	NS
Probable source of infection:			
Sporadic	13	19	NS
Post-transfusion	4	7	NS
IVDA	7	15	NS
Other	4	1	NS
HCV genotype:			
1	5	24	0.003
2	3	4	NS
3	16	8	0.002
4	3	3	NS
Mixed	0	1	NS
Not assigned	1	2	NS
Liver siderosis	9	6	NS
HGV RNA-positive (serum)	2/18	2/22	NS

IVDA, intravenous drug addicts; NS, not significant.

Statistical analysis

Whenever a serum HCV RNA level was below the sensitivity of bDNA assay, a value of 200 000 genomes/ml was arbitrarily used for calculations. All HCV RNA titers (both in serum and in liver) were Log_{10}-transformed before statistical analysis. Differences were evaluated by the Mann-Whitney's U-test. The coefficients of correlation among non-parametric variables were evaluated by the Spearman's rank correlation test. Tables of contingency were evaluated by the χ^2 method or Fisher's exact test, when appropriate.

Results

Immunocompetent chronic hepatitis C patients: virological correlations

Among the 70 immunocompetent chronic hepatitis C patients, 28 (40%) had liver steatosis with scores of 1 (18 cases), 2 (4 cases) or 3 (6 cases). Patients with or without steatosis had similar male to female ratio, mean age and route of infection (Table 1). HGV RNA was tested in 40 patients and found in the serum of 2/18 patients with steatosis and of 2/22 patients without steatosis (p=0.75) (Table 1). Steatotic patients were less likely than non-steatotic patients to be infected with HCV genotype 1 (5 *vs.* 24; p=0.003) but more likely to be infected with HCV genotype 3 (16 *vs.* 8; p=0.002) (Table 1). The other genotypes were equally distributed among the two groups of patients.

Patients with HCV genotype 3 had higher average scores of steatosis (1.33±1.2) than patients with genotype 1 (0.172±0.384; p<0.001) (Fig. 1). The difference between genotype 3 and genotype 2 (0.43±0.53) or 4 (0.5±0.55) did not reach statistical significance (P= 0.06 and 0.11, respectively) (Fig. 1).

The steatosis score did not correlate with the serum HCV RNA level (r=0.24; NS). However, a significant correlation was found between the steatosis score and the intrahepatic HCV RNA titer, both genomic-strand (r=0.26; p=0.04) (Table 2a) and the minus-strand (r= 0.36, p=0.006) (Table 2a). The statistical significance of the correlation with the titer of minus-strand (but not of the genomic-strand) further increased when only patients infected with HCV genotype 3 (r=0.64; p= 0.004) were considered, but decreased when only patients with HCV genotype 1 were analyzed (r=0.32; p= NS) (Table 2a).

Immunocompetent chronic hepatitis C patients: histological correlations

In all patients with steatosis of the liver, the fat accumulation was macrovesicular. Microvesicular steatosis was detected in 18/28 patients, but never involved more than 30% of steatotic hepatocytes. Lipogranulomata were seen in four cases. We could not establish a relationship between the prevalence of the latter two histological features and either HCV genotype or replication level (data not shown).

The degree of steatosis was correlated with the scores of lobular (r=0.62; p=0.001) and portal/periportal necroinflammation (r=0.65; p=0.0004) as well as with the fibrosis score (r=0.52; p=0.02).

Liver siderosis was found more often among patients with steatosis than among those without (9 *vs.* 6) but the difference was not significant (p=0.13). A total of 19 patients with liver steatosis (67.8%) had no liver siderosis.

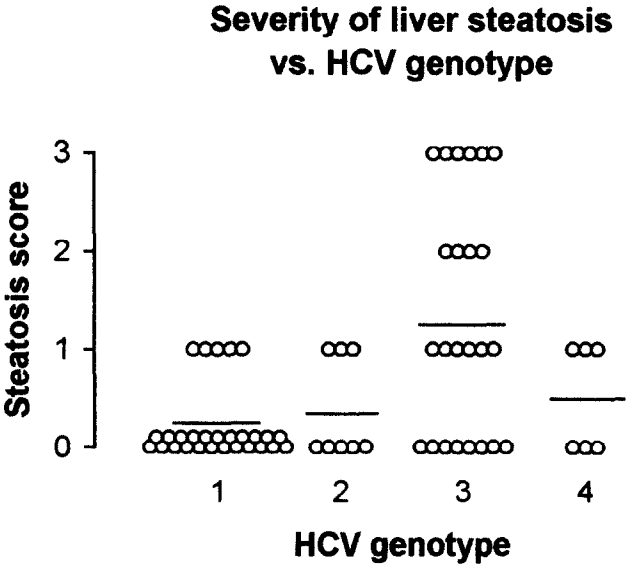


Fig. 1. Steatosis scores of 70 immunocompetent, chronic hepatitis C patients divided according to the HCV genotype. Horizontal lines denote average scores.

TABLE 2

Correlation between the liver steatosis score and the level of HCV replication in serum and liver of 70 immunocompetent chronic hepatitis C patients (a) and of 31 HCV-positive OLT recipients (b)

	Serum HCV RNA level		Intrahepatic (+) HCV RNA titer		Intrahepatic (−) HCV RNA titer	
	r	*p*	*r*	*p*	*r*	*p*
a) All immunocompetent patients	0.24	NS	0.26	0.04	0.36	0.006
Steatotic patients	0.24	NS	0.34	NS	0.49	0.01
Patients with genotype 1	0.45	NS	0.27	NS	0.32	NS
Patients with genotype 3	0.48	NS	0.37	NS	0.64	0.004
b) All HCV-positive OLT recipients	−0.02	NS	0.263	NS	0.061	NS
Steatotic patients	0.28	NS	0.492	0.005	0.111	NS
Patients with genotype 1	−0.06	NS	0.13	NS	0.11	NS
Patients with genotype 3	0.49	NS	0.514	0.03	0.49	0.04

Immunocompetent chronic hepatitis C patients: response to α-IFN

Twenty-one patients underwent α-IFN treatment. The presence of steatosis before therapy did not predict the following pattern of response (responders *vs.* non-responders: $p=0.8$; Fisher's exact test). However, all three sustained responders had moderate (1 case) to severe (2 cases) steatosis before treatment, were all infected by HCV genotype 3, and had a BMI of 20, 20 and 25. Treatment (total: 468 MU α-IFN) did not result in a significant weight loss, but the control liver biopsy performed 1 year after the end of treatment in two of them showed the absence of necroinflammation and the complete disappearance of the steatosis. The third patient refused a control liver biopsy: before treatment she had severe steatosis, and after the discontinuation of therapy her liver enzymes have been tested monthly for 1 year and found persistently normal.

Patients having undergone an OLT for HCV-related cirrhosis: virological correlations

All the 31 patients who underwent an OLT for HCV-related cirrhosis had recurrent HCV infection after OLT, as defined by the presence of detectable HCV RNA in serum. Similarly, in all patients both HCV RNA strands (i.e. the genomic strand and the replication intermediate minus-strand) were detectable in the liver, signaling graft infection by HCV.

Steatosis of the liver graft was observed in 13 patients (41.9%), with scores ranging from mild (9 cases) to moderate (1 case) to severe (3 cases). When we compared the steatosis scores with the HCV genotype, we found a correlation with the presence of genotype 3 (all HCV-positive patients: $\chi^2=24.021$, $p<0.001$; genotype 1 *vs.* 3: $\chi^2=21.609$; $p<0.001$) (Table 3). Among the 20 controls who underwent an OLT for HCV-negative liver disease, steatosis of the graft occurred in four cases (20%) and was invariably of mild degree (score 1): this difference was statistically significant with respect to HCV-infected patients ($\chi^2=41.336$; $p<0.001$) (Table 3).

Five patients, among the 28 tested, were HGV RNA-positive after OLT. The presence of HGV infection did not correlate with the occurrence of a fatty liver.

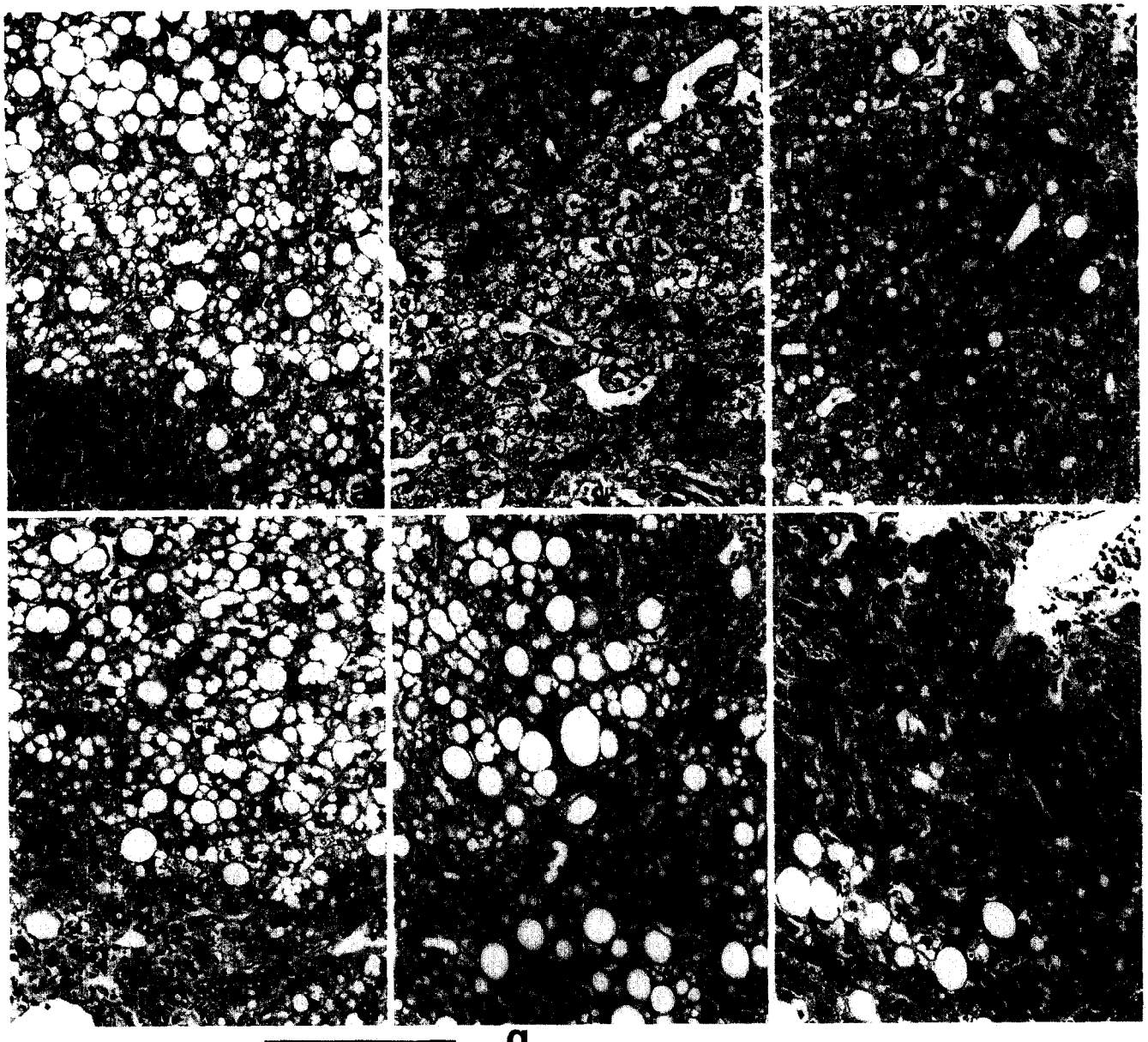
Among OLT recipients, the steatosis score did not correlate with the serum HCV RNA level ($r=-0.022$; NS) or the intrahepatic genomic- ($r=0.263$; NS) or the minus-strand HCV RNA titer ($r=0.061$; NS), as calculated on all the available liver biopsy specimens taken from all patients (Table 2b). When only the biopsy samples taken from patients who developed steatosis after OLT were considered, a significant correlation was found between the steatosis score and the titer of the intrahepatic genomic-strand HCV RNA ($r=0.492$, $p=0.005$), but not with the viremia ($r=0.283$; NS) or the intrahepatic minus-strand HCV RNA titer ($r=0.111$; NS) (Table 2b). However, when we considered only the 18 liver biopsies from patients with genotype 3, the correlation was significant for both strands of HCV RNA (genomic-strand: $r=0.514$, $p=0.03$; minus-strand: $r=0.49$, $p=0.04$) (Table 2b). The same did not apply when we considered the 43 liver biopsies from the 23 patients infected with HCV genotype 1 (Table 2b).

TABLE 3

Highest scores of liver steatosis after liver transplantation in 31 patients with HCV infection and 20 HCV-negative controls*

Score of steatosis	HCV genotype 1	HCV genotype 2	HCV genotype 3	HCV-negative
0	15	2	1	16
1	8	1	0	4
2	0	0	1	0
3	0	0	3	0

*Statistical significance: overall, $\chi^2=41.336$, $p<0.001$; only HCV-positive, $\chi^2=24.021$, $p<0.001$; HCV-1 *vs.* HCV-3, $\chi^2=21.609$, $p<0.001$.



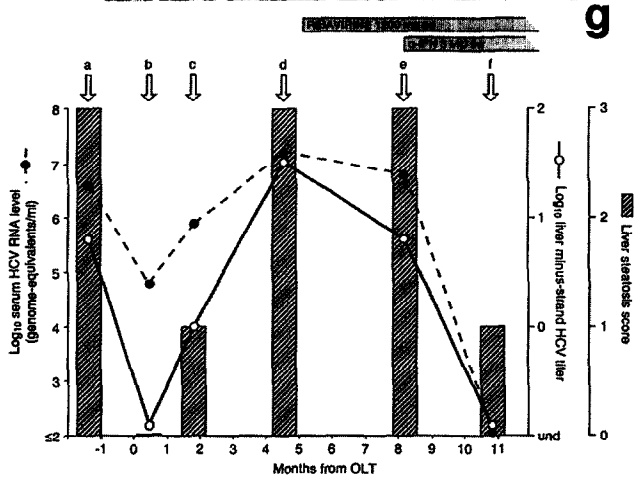


Fig. 2. Time-course of the liver score of steatosis and the HCV RNA replication level in serum (as Log_{10} genome-equivalents/ml) and liver (as Log_{10} of intrahepatic titer of minus-strand HCV RNA) in a patient who underwent OLT for end-stage HCV-related cirrhosis and received a ribavirin/α-IFN sequential treatment for recurrent hepatitis C after OLT. Panels a-f show the liver histology corresponding to the liver biopsies performed at the time-points indicated by the arrows in panel g. Other abbreviations: pd, per day; tiw, three times a week; und, undetectable.

TABLE 4

Aminoacid sequences of the HCV nucleocapsid protein of 14 liver-derived HCV RNA isolates obtained from patients infected with HCV genotypes 3 or 1 and showing different degrees of intrahepatic minus-strand HCV RNA and liver fatty infiltration

Pt #	HCV type	Liver minus-strand HCV RNA titer	Ste-atosis score	1					51				100
1	3	8192	3	MSTLPKPQRK	TKRNTIRRPQ	DVKFPGGGQI	VGGVYVLPRR	GPRLGVCATR	KTSERSQPRG	RRQPIPKARR	SEGRSWAQPG	YPWPLYGNEG	CGWAGWLLSP
2	3	512	3	----------	----------	----------	----------	----------R-----	----------	----------	----------	----------	----------
3	3	256	3	----------	----------	----------	----------	----------R-----	----------	----------	----------	----------	----------
4	3	4	0	----------	----------	----------	----------	----------R-----	----------	----------	----------	----------	----------
5	3	128	3	----------	----------	----------	----------	----------R-----	----------E	----------	----------	----------	----------
6	3	64	3	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------
7	3	64	0	----------	----------	----------	----------	----------R-----	----------E	----------	----------	----------	----------
8	3	32	3	----------	----------	----------	----------	----------R-----	----------	----------	----------	----------	----------
9	3	32	3	----------	----------	----R------	----------	----------R-----	----------	----------Q	----------	----------	----------
10	3	4	0	----------	----------	----------	----------	----------R-----	----------E	----------	----------	----------	----------
11	1	64	0	-------N----------	----------N-------	----------	----------L-------	----------R--P--	----------	----------	P-------T---------	----------	M----------
12	1	32	0	-------N----------	----------N-------	----------	----------L-------	----------R-----	----------	----------	P-------T---------	----------	M----------
13	1	32	0	-------N----------	----------N-------	----------	----------L-------	----------R-----	----------	----------	P-------T---------	----------	M----------
14	1	16	1	-------N----------	----------N-------	----------	----------L-------	----------R-----	----------	----------	P-------A---------	----------	M----------
				101					151				191
1	3	8192	3	RGSRPSWGPN	DPRRRSRNLG	KVIDTLTCGF	ADLMGYIPLV	GAPVGGVARA	LAHGVRALED	GINFATGNLP	GCSFSIFLLA	LFSCLIHPAA	S
2	3	512	3	----------	----------	----------	----------	----------	----------	----------	----------	----------	-
3	3	256	3	----------	----------	----------	----------	----------	----------	----------	----------	----------	-
4	3	4	0	----------	----------	----------	----------	----------	----------	----------	----------	----------V-------	G
5	3	128	3	----------	----------	----------	----------	----------	----------	----------	----------	----------	-
6	3	64	3	----------	----------	----------	----------	----------	----------	----------	----------	----------V-------	-
7	3	64	0	----------	----------	----------	----------	----------	----------	----------	----------	----------	-
8	3	32	3	----------	----------	----------	----------	----------	----------	----------	----------	----------	-
9	3	32	3	----------	----------	----------	----------	----------	----------	----------	----------	----------	G
10	3	4	0	----------	----------	----------	----------	----------	----------	----------	----------	----------	-
11	1	64	0	----------T	----------	----------	----------	-------L----A------	----------V------	---V---Y----------	----------	---L------TI-----S	A
12	1	32	0	----------T	----------	----------	----------	-------L----A------	----------V------	---V---Y----------	----------	---L------TI-----S	A
13	1	32	0	----------T	----------	----------	----------	-------L----A------	----------V------	---V---Y----------	----------	---L------TI-----S	A
14	1	16	1	----------T	----------	----------	----------	-------L----A------	----------V------	---V---Y----------	----------	---L------TV--TS	A

Invariant aminoacids are marked by dashes in the alignment. Only the major variant is shown in the figure. Heterogeneity was found at the following positions: aminoacid 23 (patient 9, R/K), aminoacid 47 (patients 1 and 6, C/R), aminoacid 60 (patients 2 and 3, G/E; patients 5, 7 and 10, E/G), aminoacid 191 (patients 4 and 9, G/S).

Patients having undergone an OLT for HCV-related cirrhosis: histological correlations

The steatosis occurring after OLT was invariably macrovesicular. Areas of microvesicular steatosis were seen in four patients: the area involved less than 30% of steatotic hepatocytes in three, and about 50% in one of them. Lipogranulomata were seen in two cases. These features were not correlated with the virological parameters.

The degree of steatosis was not correlated with the scores of lobular ($r=0.34$; NS), portal/periportal ($r=0.011$; NS) necroinflammation or of the fibrosis ($r=-0.09$; NS). Only four patients developed mild iron accumulation in the liver after OLT: two had also fatty accumulation, and two did not.

A recurrent hepatitis in the liver graft was observed in 23 patients out of 31 (74.2%) after an average follow-up of 40 months (range 2–116 months). The occurrence of steatosis in the graft was associated with the development of recurrent hepatitis ($p=0.035$; Fisher's exact test).

Patients having undergone an OLT for HCV-related cirrhosis: response to antiviral treatment

Fifteen of the patients with recurrent hepatitis after OLT were included in a multicenter clinical trial where ribavirin, at the daily dose of 1200 mg, p.o., was given for 3 months as monotherapy, followed by a combined regimen involving ribavirin, at the same dosage, plus recombinant α-IFN (Intron A^{TM}, Essex Schering-Plough, Luzern, Switzerland) at the dose of 3 MU, t.i.w., s.c., for 12 months (F. Negro, E. Giostra, G. Mentha, A. Hadengue, unpublished). Fig. 2g shows the time-course of the steatosis score and of the HCV RNA level (both in serum and liver) in a representative patient infected with HCV genotype 3 who received at least 3 months of combined treatment. This patient had a high score of liver steatosis before OLT (Fig. 2a). Fifteen days after OLT, his liver biopsy showed mild signs of rejection, but no steatosis (Fig. 2b). The patient developed steatosis of the liver graft 2 months after OLT (Fig. 2c), accompanied by initial signs of recurrent hepatitis. This picture was confirmed 3 months later (Fig. 2d) and justified the antiviral treatment. After 3 months of ribavirin monotherapy, both viremia and the titer of the intrahepatic minus-strand HCV RNA remained unchanged, as did the steatosis score (Fig. 2e). By contrast, after 2 months of combined regimen, the serum transaminases activities dropped, together with the serum HCV RNA level and the intrahepatic minus-strand HCV RNA titer. The liver biopsy showed a dramatic reduction of the liver steatosis (Fig. 2f). These results were paralleled in a second patient infected with HCV genotype 3 and who underwent a similar liver biopsy after 3 months of α-IFN therapy (data not shown).

Sequence analysis of the nucleocapsid-encoding region

We selected 14 patients for analyzing the complete nucleocapsid-encoding region and compared its sequence heterogeneity with the severity of steatosis and titer of minus-strand HCV RNA in the liver. Among the 10 patients with genotype 3, seven were immunocompetent chronic hepatitis C patients and three were OLT patients at the time of the maximum severity of steatosis after OLT. The four patients with genotype 1 were all immunocompetent. The steatosis scores ranged from 0 (6 cases) to 1 (1 case) to 3 (7 cases). Patients are listed in Table 4 according to the titers of intrahepatic minus-strand HCV RNA and together with the alignment of the full HCV nucleocapsid aminoacid sequences. Sequences from patients with severe steatosis, infected with HCV genotype 3 and high level of intrahepatic HCV RNA did not show any specific single aminoacid substitution, as compared with sequences obtained from patients with a low degree of or no steatosis but comparable titers of intrahepatic minus-strand HCV RNA.

Discussion

We have shown that the liver steatosis score is significantly higher in chronic hepatitis C patients infected with HCV genotype 3 than in case of infection with other viral genotypes, and that the highest levels of correlation between steatosis score and intrahepatic HCV RNA titer are reached when only patients infected with HCV genotype 3 (but not patients with genotype 1) are considered. Similarly, the occurrence of severe steatosis of the liver after OLT is associated with infection with HCV genotype 3 (but not with other genotypes), and the degree of fatty infiltration occurring after OLT again correlates with the titer of intrahepatic HCV RNA, but this, once more, only among patients with genotype 3 (and not in those with genotype 1). In the OLT patients group, the titer of both HCV RNA strands correlates with the steatosis score, while among immunocompetent patients only the minus-strand HCV RNA does. However, this discrepancy may be artefactual and due to random fluctuations of the genomic- *vs.* minus-strand ratio, as reported *in vitro* (26).

The association between HCV genotype 3 and steatosis was suggested previously (6). However, in that study, possible confounding factors which may have potentially accounted for the liver fat accumulation were not thoroughly excluded and no virological data

other than the genotype were provided. In another recent study (27), the occurrence of steatosis early after OLT was shown to be a specific hallmark of HCV reinfection, but no correlation was reported with HCV genotype and serum and/or liver viral load (27). Thus, our data provide the first direct evidence of a correlation between the level of intrahepatic HCV RNA and severity of the steatosis. Although we selected patients with BMI up to 26, thus including some with mild overweight, we still think that the liver steatosis seen in the present cases is mostly due to HCV. This assumption is not only a consequence of the virological correlations detailed above, but also of the fact that, should we consider the steatosis of our patients a mere reflection of their body mass, we would come to the paradoxical conclusion that the intrahepatic HCV RNA titer may increase according to the patient's weight.

We then examined the expression of liver steatosis in some patients treated with α-IFN and found that the virological response to treatment was paralleled by the disappearance of steatosis. Although these observations are anecdotal, they seem well documented and convincing, especially in the OLT setting.

Thus, if we put together all the above data, the steatosis of patients infected with HCV genotype 3 seems to be directly linked to the level of intrahepatic HCV RNA, and may thus represent the morphological expression of a cytopathic effect of HCV. As a result, specific HCV genome sequences, clustering in HCV genotype 3, should be responsible for the fat accumulation.

What is the HCV product responsible for the lipid accumulation? In the transgenic mouse model, the HCV core protein induced a diffuse steatosis of the liver (11). However, in that study, the fat accumulation was microvesicular, rather than macrovesicular, as in our patients. Moreover, the HCV genotype used by Moriya et al. (11) was 1b, which, according to our data and results of other authors (6), does not seem to be associated with the liver steatosis, and the level of expression of the HCV nucleocapsid protein in the liver of the transgenic mice was in general much higher than observed in the human infection. Other investigators have reported transgenic mice in which the HCV core protein expression did not result in a fatty liver (28–30).

An association between the HCV core protein and triglyceride-rich cytoplasmic fat droplets has been shown *in vitro* (31). Barba et al. (10) described the colocalization of the HCV core protein and the apolipoprotein AII by a double immunofluorescence staining followed by confocal microscopy. Since the HCV core protein binds to RNA (32), it was suggested that the liver steatosis may result from interference with the regulation of genes involved in lipid metabolism *via* binding to specific mRNA's (10). Alternatively, the HCV core protein may bind to proteins involved in the intracellular trafficking (11).

To verify the above experimental observations in the human natural infection, we selected a series of patients (infected with genotype 1 or 3) with different degrees of steatosis and intrahepatic HCV RNA titers and sequenced the HCV nucleocapsid-encoding region of the liver-derived HCV RNA. When the different aminoacid sequences were aligned, we could not assign any of them to the steatotic phenotype. The method used allows identification of the major viral variants, as the minor ones (i.e. those accounting for less than 5% of the total viral RNA) are excluded from analysis. However, given the extensive fatty infiltration, occasionally involving more than 70% of hepatocytes, we believe it unlikely that poorly represented viral variants may be responsible for the steatosis.

How can we reconcile our data with the reported, compelling evidence in favor of a role of the HCV nucleocapsid protein in the pathogenesis of the steatosis? First, we may hypothesize that the viral protein responsible for the fat accumulation in the human infection is not the nucleocapsid, and that the published data are valid only in the experimental models. To assess this hypothesis, we are now completing the full-genome sequence analysis of all of our 14 representative HCV isolates considered in the present work. Alternatively, the core protein (with particular regard to that encoded by HCV genotype 3) may be necessary, but not sufficient, to bring about the lipid accumulation. The nature of the cofactor(s) is unknown, but they may encompass either concomitant infections or host-specific factors. It is noteworthy that the development of a fatty liver in our patients did not seem to be associated with coinfection with HGV, as hinted at previously (33–35), or to a past history of intravenous drug addiction, which is the hallmark of the acquisition of multiple viral infections. Against the hypothesis of a coinfection with an unknown virus to explain the pathogenesis of steatosis, stand the experimental data obtained *in vitro* (10) and in the transgenic mouse (11). Alternatively, the polymorphism of one or more host factors may provide a more intriguing and likely mechanism. Several such factors have been described in various experimental models of steatosis and steatohepatitis, and include the tumor necrosis factor (36), the peroxisome proliferator-activated receptor alpha (37), the fatty acyl-CoA oxidase (38) and the glycerol-3-phosphate acyltransferase (39). A liver iron overload was suggested to be implicated in the steatogenesis *via*

a lipid peroxidation-mediated impairment of the intracellular trafficking (40). However, most of our steatotic patients had no liver siderosis, in agreement with others' observations (41).

Finally, the degree of the steatosis correlated with the liver disease grading and staging, at least in immunocompetent chronic hepatitis C patients. This correlation was not found in immunosuppressed OLT recipients, where the steatosis was not necessarily accompanied by a recurrent hepatitis, as reported by others (27). These findings suggest that the fat accumulation may not be an innocent and fully reversible phenomenon, but that it may instead lead to the increase of the inflammatory infiltrate as well as to the hepatocyte necrosis, as in the case of the non-alcoholic steatohepatitis, and hence to the establishment of a fibrotic liver. Since we excluded patients with moderate to severe increase of BMI, the progression to fibrosis may occur independently of significant overweight as a cofactor of a fatty liver, in contrast with a recent report (42).

In conclusion, our virological and clinical data show that the hepatocyte steatosis seen in chronic hepatitis C may be directly caused by HCV, thus representing the morphological expression of a cytopathic effect of HCV genotype 3. A derangement of the hepatocyte lipid metabolism caused by a viral protein is likely, although host protein(s) may play a role as additional cofactors.

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References

1. Seeff LB, Buskell-Bales Z, Wright EC, Durako SJ, Alter HJ, Iber FL, et al. and the National Heart, Lung, and Blood Institute Study Group. Long-term mortality after transfusion-associated non-A, non-B hepatitis. N Engl J Med 1991; 327: 1906–11.
2. Scheuer PJ, Ashrafzadeh P, Sherlock S, Brown D, Dusheiko GM. The pathology of hepatitis C. Hepatology 1992; 15: 567–71.
3. Bach N, Thung SN, Schaffner F. The histological features of chronic hepatitis C and autoimmune chronic hepatitis: a comparative analysis. Hepatology 1992; 15: 572–7.
4. Goodman ZD, Ishak KG. Histopathology of hepatitis C virus infection. Semin Liver Dis 1995; 15: 70–81.
5. Fischer HP, Willsch E, Bierhoff E, Pfeifer U. Histopathologic findings in chronic hepatitis C. J Hepatol 1996; 24: 35–42.
6. Mihm S, Fayyazi A, Hartmann H, Ramadori G. Analysis of histopathological manifestations of chronic hepatitis C virus infection with respect to virus genotype. Hepatology 1997; 25: 735–9.
7. Czaja AJ, Carpenter HA. Sensitivity, specificity and predictability of biopsy interpretations in chronic hepatitis. Gastroenterology 1993; 105: 1824–32.
8. Czaja AJ, Carpenter HA, Santrach PJ, Moore SB. Host- and disease-specific factors affecting steatosis in chronic hepatitis C. J Hepatol 1998; 29: 198–206.
9. Haruna Y, Hayashi N, Hiramatsu N, Takehara T, Hagiwara H, Sasaki Y, et al. Detection of hepatitis C virus RNA in liver tissues by an *in situ* hybridization technique. J Hepatol 1993; 18: 96–100.
10. Barba G, Harper F, Harada T, Kohara M, Goulinet S, Matsuura Y, et al. Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. Proc Natl Acad Sci USA 1997; 94: 1200–5.
11. Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, et al. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J Gen Virol 1997; 78: 1527–31.
12. Negro F, Giostra E, Krawczynski K, Quadri R, Rubbia-Brandt L, Mentha G, et al. Detection of intrahepatic hepatitis C virus replication by strand-specific semi-quantitative RT-PCR. Preliminary application to the liver transplantation model. J Hepatol 1998; 29: 1–11.
13. Negro F, Krawczynski K, Quadri R, Rubbia-Brandt L, Mondelli M, Zarski JP, et al. Detection of genomic- and minus-strand of hepatitis C virus RNA in the liver of chronic hepatitis C patients by strand-specific semi-quantitative RT-PCR. Hepatology 1999; 29: 536–42.
14. Wanless IR, Lentz JS. Fatty liver hepatitis (steatohepatitis) and obesity: an autopsy study with analysis of risk factors. Hepatology 1990; 12: 1106–10.
15. Sheth SG, Gordon FD, Chopra S. Nonalcoholic steatohepatitis. Ann Intern Med 1997; 126: 137–45.
16. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 1994; 19: 1513–20.
17. D'Alessandro AM, Kalayoglu M, Sollinger HW, Hoffmann RM, Reed A, Knechtle SG, et al. The predictive value of donor liver biopsies for the development of primary non-function after orthotopic liver transplantation. Transplantation 1991; 51: 157–63.
18. Deugnier YM, Turlin B, Powell LW, Summers KM, Moirand R, Fletcher L, et al. Differentiation between heterozygotes and homozygotes in genetic hemochromatosis by means of a histological hepatic iron index: a study of 192 cases. Hepatology 1993; 17: 30–4.
19. Turlin B, Deugnier Y. Histological assessment of liver siderosis. J Clin Pathol 1997; 50: 971.
20. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162: 156–9.
21. Nakajima-Iijima S, Hamada H, Reddy P, Kakunaga T. Molecular structure of the human cytoplasmic beta-actin gene: interspecies homology of sequences in the introns. Proc Natl Acad Sci USA 1985; 82: 6133–7.
22. Davidson F, Simmonds P, Ferguson JC, Jarvis LM, Dow BC, Follett EAC, et al. Survey of major genotypes and subtypes of hepatitis C virus using RFLP of sequences amplified from the 5′ non-coding region. J Gen Virol 1995; 76: 1197–204.
23. Linnen J, Wages J Jr, Zhang-Keck ZY, Fry KE, Krawczynski KZ, Alter H, et al. Molecular cloning and disease association of hepatitis G virus: a transfusion-transmissible agent. Science 1996; 271: 505–8.
24. Negro F, Rubbia-Brandt L, Giostra E, Seium Y, Mentha G, Quadri R, et al. Hepatitis G virus infection among liver graft recipients: anatomoclinical correlations. Dig Dis Sci 1998; 43: 2577–83.
25. Corpet F. Multiple sequence alignment with hierarchical clustering. Nucl Acids Res 1988; 16: 10881–90.

26. Shimizu YK, Iwamoto A, Hijikata M, Purcell RH, Yoshikura H. Evidence for *in vitro* replication of hepatitis C virus genome in a human T-cell line. 1992; 89: 5477–81.
27. Baiocchi L, Tisone G, Palmieri G, Rapicetta M, Pisani F, Orlando G, et al. Hepatic steatosis: a specific sign of hepatitis C reinfection after liver transplantation. Liver Transpl Surg 1998; 4: 441–7.
28. Kawamura T, Furusaka A, Koziel MJ, Chung RT, Wang TC, Schmidt EV, et al. Transgenic expression of hepatitis C virus structural proteins in the mouse. Hepatology 1997; 25: 1014–21.
29. Matsuda J, Suzuki M, Nozaki C, Shinya N, Tashiro K, Mizuno K, et al. Transgenic mouse expressing a full-length hepatitis C virus cDNA. Jpn J Cancer Res 1998; 89: 150–8.
30. Pasquinelli C, Shoenberger JM, Chung J, Chang KM, Guidotti LG, Selby M, et al. Hepatitis C virus core and E2 protein expression in transgenic mice. Hepatology 1997; 25: 719–27.
31. Moradpour D, Engler C, Wakita T, Wands JR. Characterization of cell lines allowing tightly regulated expression of hepatitis C virus core protein. Virology 1996; 222: 51–63.
32. Santolini E, Migliaccio G, La Monica N. Biosynthesis and biochemical properties of the hepatitis C virus core protein. J Virol 1994; 68: 3631–41.
33. Francesconi R, Giostra F, Ballardini G, Manzin A, Solforosi L, Lari F, et al. Clinical implications of GBV-C/HGV infection in patients with "HCV-related" chronic hepatitis. J Hepatol 1997; 26: 1165–72.
34. Petrik J, Guella L, Wight DG, Pearson GM, Hinton J, Parker H, et al. Hepatic histology in hepatitis C virus carriers coinfected with hepatitis G virus. Gut 1998; 42: 103–6.
35. Cacopardo B, Berger A, Coscntino S, Boscia V, Vinci G, Restivo R, et al. Influence of hepatitis G virus coinfection on the clinical course of chronic hepatitis C. Eur J Clin Microbiol Infect Dis 1998; 17: 709–14.
36. Pappo I, Bercovier H, Berry E, Gallilly R, Feigin E, Freund HR. Antitumor necrosis factor antibodies reduce hepatic steatosis during total parenteral nutrition and bowel rest in the rat. JPEN J Parenter Enteral Nutr 1995; 19: 80–2.
37. Fan CY, Pan J, Usuda N, Yeldandi AV, Rao MS, Reddy JK. Steatohepatitis, spontaneous peroxisome proliferation and liver tumors in mice lacking peroxisomal fatty acyl-CoA oxidase. Implications for peroxisome proliferators-activated receptor alpha natural ligand metabolism. J Biol Chem 1998; 273: 15639–45.
38. Costet P, Legendre C, More J, Edgar A, Galtier P, Pineau T. Peroxisome proliferator-activated receptor alpha-isoform deficiency leads to progressive dyslipidemia with sexually dimorphic obesity and steatosis. J Biol Chem 1998; 273: 29577–85.
39. Lanza-Jacoby S, Rosato EL. Regulatory factors in the development of fatty infiltration of the liver during gram-negative sepsis. Metabolism 1994; 43: 691–6.
40. Farinati F, Cardin R, De Maria N, Della Libera G, Marafin C, Lecis E, et al. Iron storage, lipid peroxydation and glutathione turnover in chronic anti-HCV positive hepatitis. J Hepatol 1995; 22: 449–56.
41. Haque S, Chandra B, Gerber MA, Lok ASF. Iron overload in patients with chronic hepatitis C: a clinicopathologic study. Hum Pathol 1996; 27: 1277–81.
42. Hourigan LF, Macdonald GA, Purdie D, Whitehall VH, Shorthouse C, Clouston A, et al. Fibrosis in chronic hepatitis C correlates significantly with body mass index and steatosis. Hepatology 1999; 29: 1215–9.